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Methods

Design of an eDNA sampling method for detection of an endophagous forest pest

Kathleen E. Kyle[1], Michael C. Allen[1], Nathan W. Siegert[2], Jason Grabosky[1], Julie L. Lockwood[1]

1 *Department of Ecology, Evolution and Natural Resources, Rutgers University, New Brunswick, NJ 08901, USA*
2 *US Forest Service, State, Private & Tribal Forestry, Eastern Region, Forest Health Protection, Durham, NH 03824, USA*
Corresponding author: Kathleen E. Kyle (kyle@rutgers.edu)

Abstract

Invasive wood-boring insects are a major economic and ecological concern worldwide as they impact native woody plant populations. These pest species are increasing in prevalence, with devastating impact, as global trade leads to higher rates of introduction and establishment. The emerald ash borer (*Agrilus planipennis*; EAB) is one such species, which has caused widespread damage across much of the United States and is now spreading across Europe. Non-indigenous woodborers such as EAB are difficult to detect at early stages of invasion, which is when management and eradication efforts are most effective and cost efficient. Environmental DNA (eDNA) surveys have demonstrated power in detecting invasive species when rare in the landscape due to their ability to detect trace amounts of DNA and identify to species. Here, we trialled a novel eDNA method for collecting environmental samples within host trees where invasive pest larvae are feeding, using EAB as a case study. We extracted tree cores approximately 1 cm in length using an increment hammer to assess detectability of eDNA from larvae feeding under the bark. In trees visibly infested with EAB, we observed a seasonal peak in EAB DNA detection probability (~ 64%; towards the end of the growing season), indicating a potential impact of ash tree phenology or EAB phenology on detection. When we trialled the method in a site with ash trees of low or uncertain EAB abundance, we did not record positive EAB eDNA detections. This outcome may have resulted from differing EAB phenology at the northern latitude of this survey site or because larval galleries were less numerous causing EAB DNA to be scarcer within the tree. Our results, however, provide preliminary evidence that increment hammer tree cores can be used to detect eDNA of EAB and, perhaps, other wood-boring pests. Further work is needed to clarify false negative survey detections at ash trees showing little to no signs or symptoms of infestation, as well as investigating the deposition, transport and persistence dynamics of EAB eDNA within trees.

Key words: *Agrilus planipennis*, forest management, early detection, emerald ash borer, environmental DNA, wood-boring insects

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Introduction

Phloem- and wood-boring insects are the most economically costly amongst invasive forest pests globally, resulting in over $2 billion in damage annually in the US alone (Aukema et al. 2011). They are also ecologically destructive, contributing to the functional extinction of formerly common tree species across several regions (D'Amato et al. 2023). Wood-boring insects feed and develop within their host trees, complicating early detection, eradication and suppression of invasive

populations (Liebhold et al. 2012). Environmental DNA (eDNA) surveys are now commonly used to detect aquatic invasive species and their use to detect forest pest insects is an emerging, yet growing, field of research (Valentin et al. 2020; Kirtane et al. 2022). eDNA-based sampling methods frequently yield a substantial boost in species-specific survey detection probability over conventional methods (Jerde et al. 2011; Allen et al. 2021) and are increasingly considered as part of biosecurity surveillance programmes for this reason (Trujillo-Gonzalez et al. 2020, 2022). Development of an eDNA survey that improves detection capability for wood-boring insects would improve our ability to control and eradicate newly-established invasive populations (Liebhold et al. 2016). Here, we report on a novel eDNA methodology for detection of wood-boring insects that utilises tree cores taken from host species. We illustrate the technique by evaluating its performance at detecting the presence of emerald ash borer (*Agrilus planipennis*; EAB), a highly destructive invasive woodborer of ash trees (*Fraxinus* spp.).

EAB is the most economically costly invasive insect in the United States (Aukema et al. 2011) and it has recently become established and is spreading in Europe (Volkovitsh et al. 2021). First introduced to the US in the 1990s (Siegert et al. 2014), it went undetected for at least a decade as the damage it caused was originally misidentified (Cappaert et al. 2005). Methods of detecting EAB have improved since it was initially identified in the US. Visual surveys are commonly used to identify older, high-density EAB infestations by searching for signs and symptoms, such as ash canopy dieback, epicormic sprout development, presence of adult EAB emergence holes and woodpecker damage associated with foraging on late instar EAB larvae. Other survey tools, such as girdled 'trap trees' and baited artificial traps that rely on visual and chemical attractants, have been developed (McCullough and Poland 2017; Siegert et al. 2017). The lack of a strong long-range lure for EAB, however, has consistently led to low detection rates for artificial traps, which limits their utility for identifying and delineating EAB populations and, in turn, the success of management and eradication efforts (Siegert et al. 2014; Tobin et al. 2014). Girdled 'trap trees' remain a highly effective tool for detecting low-density EAB infestations, but have the disadvantages of having to sacrifice an ash tree, lags in detection until sampling may occur at the end of the season and the need for additional resources and skills to fell and sample for EAB larvae feeding under the bark (Siegert et al. 2017). Thus, there remains a need for additional survey tools that are powerful, cheap to deploy and can be used to survey large areas for low-density EAB populations.

We posit that DNA from EAB larvae feeding within ash trees may be accessed via the collection and processing of 1 cm tree cores (Fig. 1). Our expectation stems from observations of other wood-boring and chewing insects leaving detectable amounts of their DNA on the surface of plants they utilise for food, including within the internal tunnels they create (Pirtle et al. 2021; Taddei et al. 2021; Sickel et. al. 2023). We thus expect that EAB DNA can be captured in tree cores if taken within or very close to galleries (Fig. 1). Further, if DNA from feeding EAB larvae becomes entrained in an ash tree's transport networks, it could move within the tree just as other large organic molecules and metals do (Falcone and Cooks 2016; Alvarez-Fernandez et al. 2020). If so, we suggest that EAB larval DNA may be detected in tree cores taken at distances away from galleries (Fig. 1). Finally, if EAB DNA is moving within the transport tissues (i.e. phloem and active xylem) of an ash tree, we hypothesise that the flow of DNA will follow the seasonal movement of water and nutrients within the tree; moving up towards the leaves in the spring

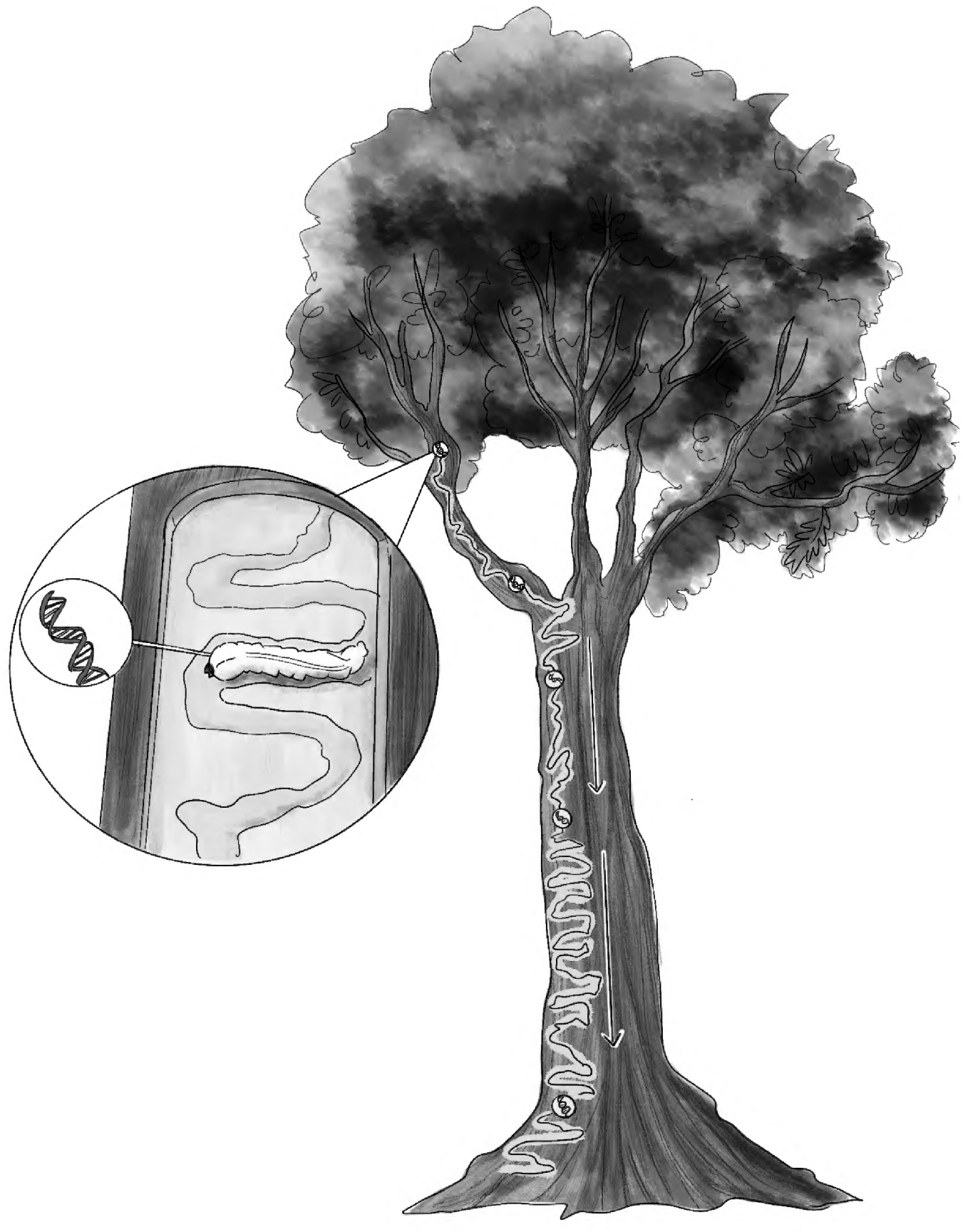

Figure 1. Schematic of our expectation that larval emerald ash borers (*Agrilus planipennis*; EAB) deposit sufficient DNA as they develop under the bark to be detectable in tree cores from within or nearby feeding galleries. EAB DNA deposited in feeding galleries may also become incorporated into the tree's transport tissues surrounding the galleries and, if so, cores collected at some presently unknown distance from a gallery may correspondingly contain detectable EAB DNA. No matter the source, we expect the availability of EAB DNA from tree cores to be highly seasonal and peak either when larvae are most actively feeding or when the host tree moves large quantities of nutrients and water to the leaves or roots – shown here by downward arrows – in the transport tissues (beginning and end of the growing season, respectively) or a combination of the two. In the present study, core samples were extracted from sample trees at breast height (1.37 m) above ground level.

and down towards the roots in the autumn for trees of temperate biomes such as ash (Mariën et al. 2019; Fig. 1). To test these hypotheses, we extracted cores from EAB-infested ash trees over the course of a single growing season and developed a quantitative polymerase chain reaction (qPCR) workflow to detect the presence of EAB DNA within these cores. We also challenged our method by collecting ash tree cores within trees that had no or low visible EAB damage and, thus, likely fewer EAB larvae and subsequently less DNA deposition.

Methods

Species-specific EAB assay design

To test our hypotheses, we first had to design a qPCR assay that could detect trace amounts of DNA from an environmental source and accurately assign species identity to EAB and no other co-occurring species. This process involves, first, identifying a candidate species-specific amplicon and then measuring assay sensitivity and specificity (Bustin and Huggett 2017). To obtain samples representative of the genetic diversity of EAB populations in North America, we sequenced the COI region from 12 EAB specimens from the epicentre of the invasion in Michigan, courtesy of the USDA APHIS PPQ, EAB Biocontrol Rearing Facility in Brighton, MI. We also collected EAB specimens from infested ash trees in Rhode Island (1), Vermont (4) and New Jersey (4); these individuals aided capture of genetic diversity across the north-eastern region of the invasive range in the US. We used a combination of adult and larval specimens, identified by specialists and preserved either via desiccation or in ethanol, respectively. We used forceps flame sterilised with 100% ethanol (EtOH) to extract one hind leg from each adult specimen along with connected muscular tissue or we cut ~ 1/3 of a larval body and placed these tissues into sterile 0.2 ml tubes. We then extracted DNA from these tissue samples using the HotSHOT method (Johnson et al. 2015) and stored extracted DNA at -20 °C until subsequent processing.

We amplified part of the COI mitochondrial DNA region via polymerase chain reaction (PCR) using primers LepF1 and LepR2 (Herbert et al. 2004). Optimised PCR cycling parameters consisted of an initial hold at 96 °C for 10 min followed by 50 cycles of denaturation at 96 °C for 30 seconds, annealing at 44 °C for 40 seconds and extension at 72 °C for 45 seconds, with a final extension step at 72 °C for 5 minutes. DNA was amplified in 20 µl reactions consisting of 1X PCR buffer II, 2.75 mM $MgCl_2$, 250 pM each dNTP, 200 nM of each primer and 1.5U of Taq Polymerase Gold. We ran all PCRs on a Veriti 96-Well Thermal Cycler (Applied Biosystems, Life Technologies, Carlsbad, CA) and visualised reactions in 1% agarose gel with gel red dye. Successful reactions yielded amplicons roughly 300 bp in size that were cleaned using the ExoSAP-IT enzyme (Carlsbad, CA). Then, approximately 10 ng of cleaned template DNA was mixed with 25 pmoles of each primer and sent for bidirectional Sanger sequencing (GenScript, Piscataway NJ). Using *Geneious Prime* (version 2021.0.1), we trimmed the sequences, combined them with 35 additional EAB sequences from GenBank (Accession numbers provided in Appendix 1) and aligned them to identify a consensus sequence that was conserved across individuals of the species. We then used *Primer Express* (version 3.0.1) to identify a TaqMan qPCR primer and probe set optimal for amplifying a segment of this conserved region, hereafter referred to as "EAB COI assay".

All qPCR reactions consisted of 500 nM each primer, 250 nM probe, 1× TaqMan® Environmental Mastermix II with no UNG and 2 µl DNA. The optimised reaction protocol included an initial denaturing step of 96 °C for 10 min, followed by 45 cycles of denaturation at 96 °C for 15 s and annealing and extension at 60 °C for 1 min. All reactions were run on an Applied Biosystems StepOne Plus Real-Time PCR System (Applied Biosystems, Life Technologies, Carlsbad, CA). Each sample was tested for the presence of EAB eDNA in triplicate and considered positive if at least one of three technical replicates successfully amplified.

High-density EAB infestation: Tree core sampling through time

To investigate whether larval EAB DNA was present in tissues of ash trees, we took core samples (containing cambium, phloem and xylem) from green ash (*Fraxinus pennsylvanica*) throughout the 2021 growing season at two sites in New Brunswick, New Jersey, USA. We sampled 7–13 trees per day on 13 dates between 21 May and 15 October (Table 1). We chose initial sampling dates to coarsely coincide with growing degree day (GDD) accumulation thresholds for major EAB life history events. Adult emergence begins around 450 GDD – base 50 °F (10 °C) – and peaks around 1100 GDD (Siegert et al. 2015). As such, we estimated that our first sampling event, in May, was during the EAB pupal period; our second event, in June, coincided with peak EAB adult emergence; and our last weeks of sampling, from August through October, were when larvae had hatched from eggs, burrowed into trees and begun feeding. We elected to perform weekly sampling during this later time-frame assuming that EAB DNA may be deposited at higher rates by larvae feeding within galleries. In addition, EAB DNA could be transported within vascular tissues to the base of the trees during autumn senescence (Alvarez-Fernandez et al. 2014; Falcone and Cooks 2016).

Core samples were taken at breast height (~ 1.37 m above the ground) using a Haglöf 2.5 cm (1-inch) increment hammer at ash trees ranging from ~ 15 to 90 cm diameter at breast height (dbh). All trees showed visible signs of EAB colonisation (e.g., canopy dieback, epicormic sprouting); however, no bark splits or adult exit holes were visibly evident within 1 m of locations where we extracted tree cores. We were not able to strip bark from our sample trees after core extractions as they were on private property and we also wished to resample the same trees. Thus, we cannot be sure how close core samples were to larval feeding galleries or pupation chambers. Before taking each sample, we flame-sterilised the cutting tube of the increment hammer using 100% EtOH to remove any surface DNA between sample collections. At each tree, we took two 1 cm core samples (one each from north

Table 1. New Jersey core collection dates along with ordinal date and accumulated growing degree days and sample size for each sampling date.

Calendar Date (2021)	Ordinal Date	GDD Accumulation Base 50 °F (10 °C)	Sample Size (n)
21 May	141	366	20
30 June	181	1185	20
6 Aug	218	2105	14
13 Aug	225	2307	24
20 Aug	232	2504	22
27 Aug	239	2702	22
3 Sept	246	2853	16
10 Sept	253	2990	26
17 Sept	260	3148	26
24 Sept	267	3284	26
1 Oct	274	3367	26
8 Oct	281	3474	20
15 Oct	288	3584	20

and south aspects). We placed the core samples into tissue disruption tubes for later DNA extraction. In total, we took 282 cores from 21 ash trees. On each sampling date, we also extracted 1-cm cores (n = 2 or 4, depending on the number of sample sites) from nearby oak trees (*Quercus* spp.) expecting that these could serve as 'field negative control' cores where we would not expect EAB DNA to be present.

In the laboratory, we extracted DNA from core samples using the DNeasy Plant Pro Kit (Qiagen) following manufacturer's protocols and tested all samples via qPCR with our EAB COI assay as described above. Each DNA extraction and qPCR run included negative controls to ensure no in-lab contamination occurred.

Low-density EAB infestation: Tree core sampling across varying infestation levels

On 22–23 September 2021 (ordinal dates 265–266), we collected tree cores from white ash trees (*Fraxinus americana*) in Loudon, New Hampshire, USA to explore the ability of the method to detect EAB when present at lower densities. This EAB infestation was much less advanced than at our New Jersey sampling sites. Host trees at our New Hampshire sites exhibited a wide array of decline, characterised on a spectrum from no visible signs of infestation ('no damage') to minimal signs of infestation ('light damage') to a small degree of dieback and epicormic sprouting ('moderate damage'). Samples were taken using the same tree core methods as described above. Accumulated growing degree days at this site during the two-day sampling effort were 2224 and 2245. We collected samples from a total of 30 trees, 10 each from three tree damage categories: no damage, light damage and moderate damage. We extracted four tree cores, one from each cardinal direction, from each tree to increase probability of EAB detection. In total, 120 cores were taken from ash trees. Negative control cores were also taken from nearby birch trees (*Betula* spp.) to confirm complete decontamination of the increment hammer between samples.

Statistical analysis: Tree core sampling through time

We fitted a Bayesian generalised additive model (GAM) with a Bernoulli error distribution and a logit link function to describe the phenology of EAB eDNA detection over the course of the 2021 sampling season in New Jersey, as well as to assess the effects of covariates on detection rates (GDD accumulation and side of tree). Additionally, due to potential contamination (i.e. amplification of small quantities of EAB DNA) found in some negative control samples (see Results), we also conducted a parallel and more conservative set of analyses that were identical to those described above, but that only treated samples with at least two of the three qPCR technical replicates amplifying to be true detections (see Appendix 2). As it was visually evident that all 21 trees sampled were infested with EAB (see above), all detections and non-detections were assumed to provide information for the probability of detection given presence. The dependent variable was eDNA detection (1) or non-detection (0), based on the qPCR assay results for each sample (i.e. '1' if any of the qPCR technical replicates for a sample amplified EAB DNA, '0' otherwise). Independent variables in the model included a random effect of sample tree and two fixed effects: a binary variable indicating side of tree (north or south) and a continuous variable for the number of accumulated growing degree days (GDD) on the date of sampling. GDD data were obtained from the Cornell

Network for Environment and Weather Applications (NEWA). We used vague priors on all parameters and ran four chains of 4000 iterations each, including 2000 discarded warm-up iterations. Convergence was assessed using Gelman-Rubin statistics (rhat < 1.1). We compared models with different combinations of independent variables, based on leave-one-out information criterion (LOOIC).

We used the modelled detection probabilities from the GAM (i.e. the posterior distributions) to conduct an additional analysis to estimate how many core samples would be required to detect EAB in an infested tree with 95% certainty. To do this, we used the formula $p^* = 1-(1-p)^n$, where p^* is the probability of obtaining at least one positive core sample, p is the modelled per-sample detection probability and n is the number of core samples taken. We set p^* equal to 0.95 and solved for n to estimate the number of cores required for each sampling event to have a 95% probability of detecting EAB presence.

Results

Species-specific EAB assay design

Based on the sequences we generated and those publicly available on GenBank, we designed primers EAB_COI-F (TTCGAGCAGAATTAGGAAATCCA) and EAB_COI-R (AAGCATGAGCAGTAACAATAACATTATAGA) and probe EAB_COI-Probe (CATTAATTGGCAATGACC), which target a 78 bp fragment within the COI mtDNA region of EAB. Our specificity testing indicated that our assay was specific to our target species (see Appendix 1). Based on these tests, we assume that any core samples that amplified with the EAB COI assay indicate EAB individuals living within sampled trees.

High-density EAB infestation: Tree core sampling through time

Of the 282 tree cores collected over the 2021 growing season, 120 tested positive for EAB DNA. Comparison of the GAMs explaining EAB eDNA detection probability indicated that the model including only the random effect of tree and the fixed effect of GDD was most parsimonious, as indicated by LOOIC comparison of models (Table 2; Fig. 2). The side of the tree where core samples were taken (north vs. south) showed little influence on detection probability ($\beta = 0.07$, 95% CI = [-0.46, 0.58] from the model including this variable plus GDD). The model revealed a hump-shaped temporal pattern in the proportion of samples testing positive for

Table 2. Comparison of Bayesian generalised additive models (GAMs) describing the phenology of emerald ash borer tree core eDNA detection probability, based on leave-one-out information criterion (LOOIC).

Model	ΔELPD[a]	ΔELPD SE	ELPD	LOOIC
Tree + GDD	0.0	0.0	-176.4	352.8
Tree + GDD + direction	-0.8	0.3	-177.2	354.4
Tree	-13.2	5.8	-189.6	379.2
Tree + direction	-14.4	5.8	-190.8	381.6

[a] Difference in ELPD (expected log pointwise predictive density) between the model and the model with the highest ELPD; higher values represent more parsimonious models.

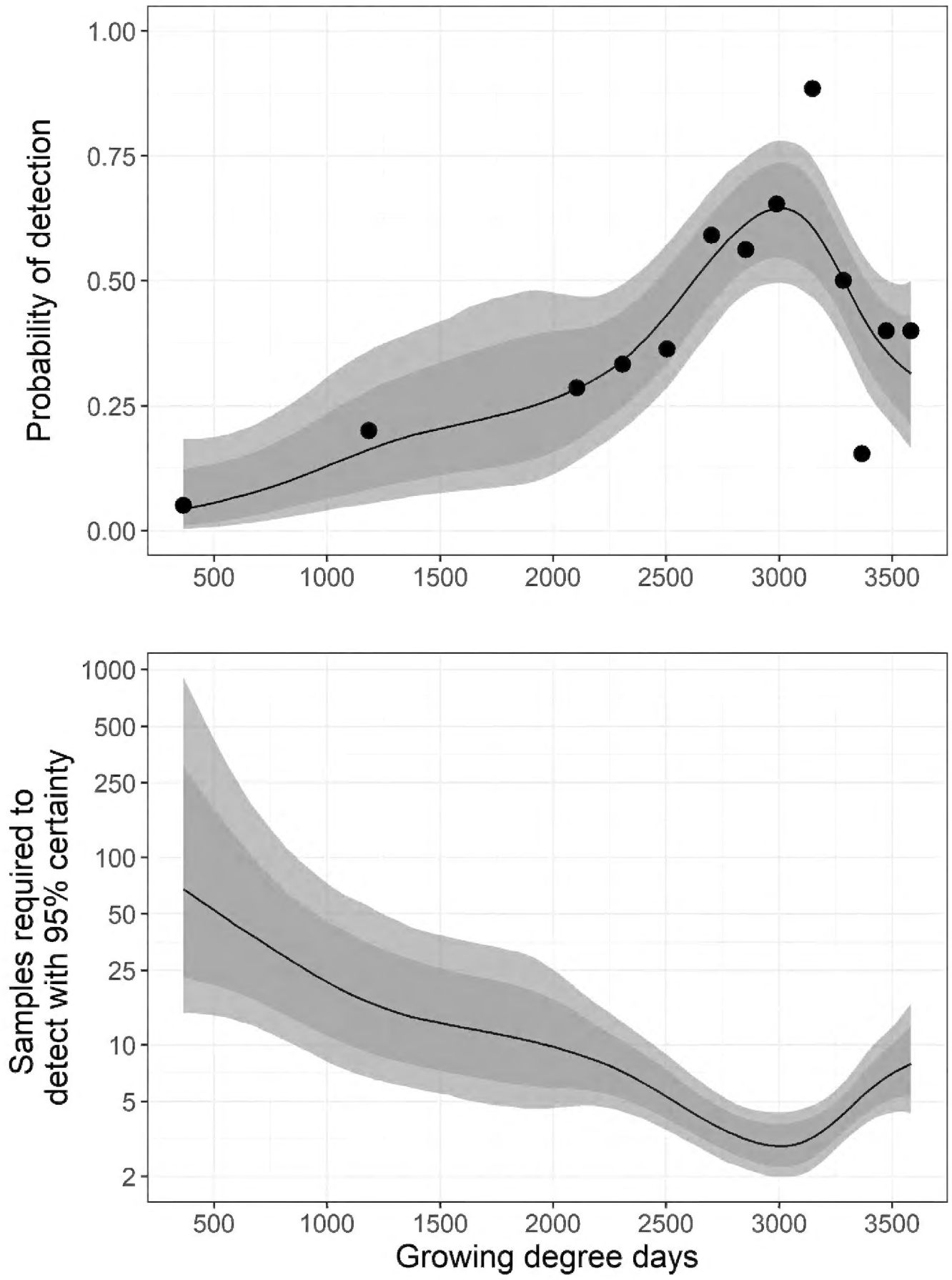

Figure 2. Top: probability of detecting eDNA from emerald ash borer (*Agrilus planipennis*; EAB) using the tree core sampling method at sites in New Jersey, USA over time. Bottom: the number of ash tree core samples needed per tree to ensure 95% confidence in detection of EAB larval presence within a tree. Time is represented as growing degree day (GDD) accumulation, base 50 °F (10 °C). Black lines represent the posterior medians from a Bayesian general additive model (GAM); grey shading indicates 80% and 95% credible intervals. Black circles show the proportion of samples with detections on each sampling date calculated from the raw data.

EAB DNA as growing degree days accumulated, with peak detection probability occurring at ~ 3000 GDD (Fig. 2). After this peak, the proportion of positive core samples dropped off, indicating that, at least in New Jersey, a sampling date around mid-September is optimal to collect ash tree core samples to detect the presence of EAB larvae within an infested ash tree. The temporal trends observed in detection probability were clearly reflected in the estimates of the number of cores required to obtain an EAB detection with 95% certainty (Fig. 2). This analysis revealed that, at peak detection probability (~ 3000 GDD), roughly 2.5 core samples per tree would be required to detect EAB larvae within a tree with 95% confidence (Fig. 2).

None of our laboratory negative controls revealed contamination. However, three of our 30 'negative control' *Quercus* tree cores returned weak positive EAB detections. These samples had cycle threshold (Ct) values of 39–41 and all had only one of three technical replicates amplify. The parallel analysis of detection probability that used stricter guidelines for declaring samples as 'positive' for EAB DNA revealed very similar phenological patterns (Appendix 2: Fig. A2).

Low-density EAB infestation: Tree core sampling across varying infestation levels

Of the 120 core samples we took from ash trees at the lower EAB density site in New Hampshire, we recorded no positive qPCR detections of EAB. All negative controls also returned negative qPCR results. This sampling effort took place at ~ 2200 accumulated GDD, potentially missing the point when peak detection was witnessed during our high-density sampling season that took place at a more southern latitude.

Discussion

Our results indicate that DNA deposited by EAB larvae can be recovered from ash tree cores using a standard increment hammer and confirmed via species-specific qPCR detection. We also show that there may be strong seasonality to this method of EAB detection. This seasonal pattern follows our hypothesis that, as they feed, EAB larvae deposit their DNA within galleries or it becomes incorporated within ash tree tissues. Further, the peak in seasonal eDNA detection probability of larvae was about three months later than the peak trap catch dates in conventional survey methods for capturing adults (Tobin et al. 2021). The optimal timing for conventional trapping methods is considerably earlier in the growing season (~ 1000 accumulated GDD, which corresponds to late June in New Jersey; Tobin et al. (2021)). This result suggests the potential utility of eDNA for survey season extension. Our results represent a novel methodology for detecting the presence of wood-boring insect larvae within their host trees and may prove particularly useful for detection of invasive insects within forested landscapes or urban environments more broadly. However, our results were less encouraging regarding the detection of EAB when present within ash trees at low enough abundance that the trees exhibited little visual evidence of infestation. These mixed results suggest several avenues for continued research, which we detail below.

Phenology is an important consideration in any population assessment method (e.g. Kean and Stringer (2019)) and our results showed that this concept also applies to eDNA detection via tree core surveys. We show that, if cores are taken at the optimal time of year, then eDNA survey detection rates can be quite high: ~ 64% per core sample for EAB at their peak; or, requiring only 2–3 cores per infested tree to detect EAB with 95% confidence. Conversely, like conventional methods, if samples are taken at the wrong time of year, detection rates can be very low or require a prohibitively large number of samples to confirm EAB larval presence. It is unknown, without a head-to-head comparison, whether eDNA detection rates are higher or lower than conventional tools and how they perform relative to one another when EAB are present at very low densities. However, the relative immediacy and ease of collecting eDNA core samples (e.g. few to no revisits or tree girdling and debarking required) alone make this a potentially attractive survey tool. Our method provides logistic ease that conventional approaches may not. For example, one person in the field can

easily take tree cores and store them for later processing, moving and sampling over a wide spatial extent in a single day. Samples can then be processed quickly in the lab using our qPCR approach or our methods can be adapted for use in other molecular workflows that vary in their immediacy and sensitivity (e.g. field-based qPCR, LAMP, digital droplet PCR). However, the relative feasibility of each workflow requires independent evaluation. For instance, the extraction of DNA from wood is not as straightforward as it is from frass or tissue and may be difficult to accomplish in the field, potentially increasing turnaround time from sample collection to result. This is certainly another research front that needs exploring for this method to be adopted on a wide scale. Though given how rapidly eDNA technologies are presently advancing, it is likely that improved methods for DNA extraction and more sensitive molecular techniques will aid to expedite the vetting process of this method.

The exact causes for the seasonality we observed in tree core eDNA detection rates are unknown. If EAB eDNA is indeed transported throughout the tree along with water and nutrients, the role of tree physiology becomes a key consideration in survey design. The ecophysiology of sap flow in trees has a long research history (e.g. Zimmermann (1957)) and could aid in optimising timing for when to conduct tree core eDNA surveys and possibly help identify within-tree sampling locations across seasons (e.g. leaves, twigs, trunk, roots). In contrast, the dynamics of DNA deposition by endophagous insects within host plants is a new field of study (e.g. Pirtle et al. (2021)). While there is a growing research base that has investigated the movement and degradation of eDNA in aquatic systems (Strickler et al. 2015) and on surfaces within terrestrial systems (Valentin et al. 2021), very little is known about how long arthropod DNA may persist within larval galleries or transport tissues of trees. This DNA is protected from UV degradation, which should allow it to persist in a detectable state for long periods of time (Valentin et al. 2021). However, it may also experience a suite of thermal, chemical and biological interactions within trees and their tissues that may degrade the DNA, limiting the time-frame over which it can be detected by a molecular assay. As with other eDNA techniques, an in-depth understanding on the 'ecology' of arthropod eDNA under bark and within transport tissues of trees is needed before our tree core approach can be widely adopted for use in invasive species management.

Nutrient flow within ash trees is restricted when larval EAB densities are high because larval galleries serve to interrupt phloem movement (Flower et al. 2018). This pattern suggests that the high eDNA detection rates found within highly-damaged trees may only reflect a 'pooling' of EAB DNA in tree tissues near larval galleries. Our tree cores may be capturing EAB DNA from these pools and/or from the inner surfaces of larval tunnels, which are rich in DNA from frass and chewing of EAB larvae (Pirtle et al. 2021; Taddei et al. 2021). When trees are healthier, any deposited EAB DNA may be transported over much more of the tree making it less likely to be captured when extracting only a small number of tree cores. Similarly, trees with low densities of EAB larvae will have fewer galleries making it less likely that any core sample taken will include tissues from gallery tunnels. These possibilities may explain, in part, our failure to detect EAB DNA within cores taken from trees with lower visual evidence of damage. It is also possible that the timing of our low-density sampling effort was offset enough from the autumnal senescence of these more northern ash trees that we simply missed the detection peak there. We witnessed overall much lower GDD accumulation at our low-density sampling location (~ 2200 GDD) than when the probability of detection peaked in our high-density study (~ 3000 GDD).

However, there is also a suite of other factors that impact the onset of autumnal senescence aside from GDD accumulation that could point towards the differing 'peak' detection timings at these different latitudes (Gill et al. 2015).

The extent to which the phenology of tree core eDNA detection does not overlap with the phenology of detection from conventional methods suggests an opportunity to use this new tool, once fully vetted, to extend the window of EAB detection for delimiting surveys or landscape-scale surveillance. Additionally, this combination of eDNA and conventional trapping programmes may apply to invasive wood-boring insects more generally, as most traps effectively catch the ephemeral flying adult stage of a target species, but are ineffective at detecting larval stages, which our eDNA tree core method could be well-suited to do. The eDNA tree core method could also complement other forms of EAB surveys besides traps, for example, by concentrating tree core eDNA sampling activity on or near girdled 'trap trees', which represent the best-known attractant for the species (Siegert et al. 2017). For optimal management of EAB, having multiple well-tested tools that target a range of developmental stages is desirable – especially at invasion fronts such as Oregon, USA or western Europe – and also for other damaging wood-boring species (e.g. Asian long-horned beetle, *Anoplophora glabripennis*; Taddei et al. (2021)).

Our study introduces a promising and novel survey tool for detecting invasive wood-boring insects, along with proof-of-concept testing and insights into its performance. The key to realising if and when this method provides benefits to detecting EAB or other invasive wood-boring forest insects is: (1) executing an explicit test of the sampling effort necessary to detect EAB across different larval abundances; (2) a controlled repeat of our methods across a more inclusive array of EAB densities and latitudes to identify seasonal peaks and the degree to which these peaks diminish with lower EAB infestation levels; (3) identification of how much higher or lower detection probability of EAB is as a core sample is taken at varying distances away from larval feeding galleries and 4) a side by side comparison of landscape-level detection probability with the tree core method versus traditional trapping and detection techniques. It will only be possible for this tool to become operational after investigating these questions to better provide information about the power of the method as well as the potential shortcomings. Given the impacts of invasive wood-boring insects and the increasing number of invasions that are occurring globally (Aukema et al. 2011), having an effective and logistically simple sampling tool to increase detection probabilities for these species would contribute to preventing and mitigating the damage they cause to trees and forest ecosystems worldwide.

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Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

No ethical statement was reported.

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Author contributions

All authors have contributed equally.

Author ORCIDs

Kathleen E. Kyle https://orcid.org/0000-0001-8830-4460
Michael C. Allen https://orcid.org/0000-0002-6632-4337
Jason Grabosky https://orcid.org/0000-0003-4226-2888
Julie L. Lockwood https://orcid.org/0000-0003-0177-449X

Data availability

The protocol for this newly-designed eDNA sampling method has been published on our lab environmental DNA website (https://sites.rutgers.edu/edna/). All sequences we generated during this study will be publicly available on GenBank (Accession numbers PP373086–PP373114).

References

Allen MC, Nielsen AL, Peterson DL, Lockwood JL (2021) Terrestrial eDNA survey outperforms conventional approach for detecting an invasive pest insect within an agricultural ecosystem. Environmental DNA 3(6): 1102–1112. https://doi.org/10.1002/edn3.231

Alvarez-Fernandez A, Diaz-Benito P, Abadia A, Lopez-Millan AF, Abadia J (2014) Metal species involved in long distance metal transport in plants. Frontiers in Plant Science 5. https://doi.org/10.3389/fpls.2014.00105

Aukema JE, Leung B, Lovacs K, Chivers C, Britton KO, Englin J, Frankel SJ, Haight RG, Holmes TP, Liebhold AM, McCullough DG, Von Holle B (2011) Economic impacts of non-native forest insects in the continental United States. PLOS ONE 6(9): e24587. https://doi.org/10.1371/journal.pone.0024587

Bürkner PC (2017) brms: An R package for Bayesian multilevel models using Stan. Journal of Statistical Software 80(1): 1–28. https://doi.org/10.18637/jss.v080.i01

Bustin S, Huggett J (2017) qPCR primer design revisited. Biomolecular Detection and Quantification 14: 19–28. https://doi.org/10.1016/j.bdq.2017.11.001

Cappaert D, McCullough DG, Poland TM, Siegert NW (2005) Emerald ash borer in North America: A research and regulatory challenge. American Entomologist (Lanham, Md.) 51(3): 152–165. https://doi.org/10.1093/ae/51.3.152

D'Amato AW, Orwig DA, Siegert NW, Mahaffey A, Benedict L, Everett T, Daigle J, Catanzaro P, Cusack C (2023) Species Preservation in the Face of Novel Threats: Cultural, Ecological, and Operational Considerations for Preserving Tree Species in the Context of Non-Indigenous Insects and Pathogens. Journal of Forestry 121(5–6): 470. https://doi.org/10.1093/jofore/fvad024

Falcone CE, Cooks RG (2016) Molecular recognition of emerald ash borer infestation using leaf mass spectrometry. Rapid Communications in Mass Spectrometry 30(11): 1304–1312. https://doi.org/10.1002/rcm.7561

Flower CE, Lynch DJ, Knight KS, Gonzalez-Meler MA (2018) Biotic and abiotic drivers of sap flux in mature green ash trees (*Fraxinus pennsylvanica*) experiencing varying levels of emerald ash borer (*Agrilus planipennis*) infestation. Forests 9(6): 301. https://doi.org/10.3390/f9060301

Gill AL, Gallinat AS, Sanders-DeMott R, Rigden AJ, Gianotti AJS, Mantooth JA, Templer PH (2015) Changes in autumnal senescence in northern hemisphere deciduous trees: A meta-analysis of autumn phenology studies. Annals of Botany 116(6): 875–888. https://doi.org/10.1093/aob/mcv055

Herbert PD, Penton EH, Burns JM, Janzen DH, Hallwachs W (2004) Ten species in one: DNA barcoding reveals cryptic species in the neotropical skipper butterfly *Astraptes fulgerator*. Proceedings of the National Academy of Sciences of the United States of America 101(41): 14812–14817. https://doi.org/10.1073/pnas.0406166101

Jerde CL, Mahon AR, Chadderton WL, Lodge DM (2011) "Sight-unseen" detection of rare aquatic species using environmental DNA. Conservation Letters 4(2): 150–157. https://doi.org/10.1111/j.1755-263X.2010.00158.x

Johnson BJ, Robson MG, Fonseca DM (2015) Unexpected spatiotemporal abundance of infected Culex restuans suggest a greater role as a West Nile virus vector for this native species. Infection, Genetics and Evolution 31: 40–47. https://doi.org/10.1016/j.meegid.2015.01.007

Kean JM, Stringer LD (2019) Optimising the seasonal deployment of surveillance traps for detection of incipient pest invasions. Crop Protection (Guildford, Surrey) 123: 36–44. https://doi.org/10.1016/j.cropro.2019.05.015

Kirtane A, Dietschler NJ, Bittner TD, Lefebvre MB, Celis S, O'Connor K, Havill N, Whitmore MC (2022) Sensitive environmental DNA (eDNA) methods to detect hemlock wooly adelgid and its biological control predators *Leucotaraxis* silver flies and a *Laricobius* beetle. Environmental DNA 4(5): 1136–1149. https://doi.org/10.1002/edn3.317

Klymus KE, Merkes CM, Allison MJ, Goldberg CS, Helbing CC, Hunter ME, Jackson CA, Lance RF, Mangan AM, Monroe EM, Piaggio AJ, Stokdyk JP, Wilson CC, Richter CA (2020) Reporting the limits of detection and quantification for environmental DNA assays. Environmental DNA 2(3): 271–282. https://doi.org/10.1002/edn3.29

Liebhold AM, Brockerhoff EG, Garrett LJ, Parke JL, Britton KO (2012) Live plant imports: The major pathway for forest insect and pathogen invasions of the US. Frontiers in Ecology and the Environment 10(3): 135–143. https://doi.org/10.1890/110198

Liebhold AM, Berec L, Brockerhoff EG, Epanchin-Niell RS, Hastings A, Herms DA, Kean JM, McCullough DG, Suckling DM, Tobin PC, Yamanaka T (2016) Eradication of Invading Insect Populations: From Concepts to Applications. Annual Review of Entomology 61(1): 335–352. https://doi.org/10.1146/annurev-ento-010715-023809

Mariën B, Balzarolo M, Dox I, Leys S, Lorène MJ, Geron C, Portillo-Estrada M, AbdElgawad H, Asard H, Campioli M (2019) Detecting the onset of autumn leaf senescence in deciduous forest trees of the temperate zone. The New Phytologist 224(1): 166–176. https://doi.org/10.1111/nph.15991

McCullough DG, Poland TM (2017) Building Double-decker Traps for Early Detection of Emerald Ash Borer. Journal of Visualized Experiments 128: e55252. https://doi.org/10.3791/55252-v

Pirtle EI, van Rooyen AR, Maino J, Weeks AR, Umina PA (2021) A molecular method for biomonitoring of an exotic plant-pest: Leafmining for environmental DNA. Molecular Ecology 30(19): 4913–4925. https://doi.org/10.1111/mec.16092

Sickel W, Kulow J, Krüger L, Dieker P (2023) BEE-quest of the nest: a novel method for eDNA-based, nonlethal detection of cavity-nesting hymenopterans and other arthropods. Environmental DNA 5(6): 1163–1176. https://doi.org/10.1002/edn3.490

Siegert NW, McCullough DG, Liebhold AM, Telewski FW (2014) Dendrochronological reconstruction of the epicentre and early spread of emerald ash borer in North America. Diversity & Distributions 20(7): 847–858. https://doi.org/10.1111/ddi.12212

Siegert NW, McCullough DG, Andresen JA (2015) Growing Degree Day Information. Michigan State University. http://www.ipm.msu.edu/agriculture/christmas_trees/growing_degree_day_information

Siegert NW, McCullough DG, Poland TM, Heyd RL (2017) Optimizing Use of Girdled Ash Trees for Management of Low-Density Emerald Ash Borer (Coleoptera: Buprestidae) Populations. Journal of Economic Entomology 110(3): 1096–1106. https://doi.org/10.1093/jee/tox092

Strickler KM, Fremier AK, Goldberg CS (2015) Quantifying effects of UV-B, temperature, and pH on eDNA degradation in aquatic microcosms. Biological Conservation 183: 85–92. https://doi.org/10.1016/j.biocon.2014.11.038

Taddei A, Becker M, Berger B, Da Lio D, Feltgen S, König S, Hoppe B, Rizzo D (2021) Molecular identification of Anoplophora glabripennis (Coleoptera: Cerambycidae) and detection from frass samples based on real-time quantitative PCR. Journal of Plant Diseases and Protection 128(6): 1587–1601. https://doi.org/10.1007/s41348-021-00501-7

Tobin PC, Kean JM, Suckling DM, McCullough DG, Herms DA, Stringer LD (2014) Determinants of successful arthropod eradication programs. Biological Invasions 16(2): 401–414. https://doi.org/10.1007/s10530-013-0529-5

Tobin PC, Strom BL, Francese JA, Herms DA, McCullough DG, Poland TM, Ryall KL, Scarr T, Silk PJ, Thistle HW (2021) Evaluation of trapping schemes to detect emerald ash borer (Coleoptera: Buprestidae). Journal of Economic Entomology 114(3): 1201–1210. https://doi.org/10.1093/jee/toab065

Trujillo-Gonzalez A, Becker JA, Huerlimann R, Saunders RJ, Hutson KS (2020) Can environmental DNA be used for aquatic biosecurity in the aquarium trade? Biological Invasions 22(3): 1011–1025. https://doi.org/10.1007/s10530-019-02152-0

Trujillo-Gonzalez A, Thuo DN, Divi U, Sparks K, Wallenius T, Gleeson D (2022) Detection of khapra beetle environmental DNA using portable technologies in Australian biosecurity. Frontiers in Insect Science 2: 795379. https://doi.org/10.3389/finsc.2022.795379

Valentin RE, Fonseca DM, Gable S, Kyle KE, Hamilton GC, Nielsen AL, Lockwood JL (2020) Moving eDNA surveys onto land: Strategies for active eDNA aggregation to detect invasive forest insects. Molecular Ecology Resources 20(3): 746–755. https://doi.org/10.1111/1755-0998.13151

Valentin RE, Kyle KE, Allen MC, Welbourne DJ, Lockwood JL (2021) The state, transport and fate of aboveground terrestrial arthropod eDNA. Environmental DNA 3(6): 1081–1092. https://doi.org/10.1002/edn3.229

Volkovitsh MG, Bieńkowski AO, Orlova-Bienkowskaja MJ (2021) Emerald ash borer approaches the borders of the European union and Kazakhstan and is confirmed to infest European ash. Forests 12(6): 691. https://doi.org/10.3390/f12060691

Zimmermann MH (1957) Translocation of Organic Substances in Trees. II. On the Translocation Mechanism in the Phloem of White Ash (*Fraxinus Americana* L.). Plant Physiology 32(5): 399–404. https://doi.org/10.1104/pp.32.5.399

Appendix 1

EAB COI qPCR assay design, specificity and sensitivity testing

Methods

For the design of our EAB COI assay, we generated 21 sequences from specimens collected in the species' invasive range and combined with 35 sequences that were publicly available on GenBank. The GenBank accession numbers for those sequences are listed below:

AY756137, AY864194, DQ861319, DQ861320, GU013563, JF887747, KM845113, KT250461, KT250462, KT250467, KT250473, KT250476,

KT250479, KT250480, KT250487, KT250490, KT250504, KT250508, MF286180, MG477998, MH159080, MH159105, MN548248–MN548260.

We conducted *in silico* specificity testing on 10 closely-related sympatric species to ensure none would cross-amplify with the assay we designed. We also performed a nucleotide blast in GenBank to ensure no published sequences had high percent identity with our target amplicon sequence. Finally, we conducted *in vitro* specificity testing on seven co-occurring Coleoptera with specimens trapped together at EAB invasion sites in Connecticut.

We evaluated our assay's lower limits of detection (LOD) by creating an 8-level 10-fold dilution series using genomic DNA (gDNA) extractions from EAB specimen legs with attached muscle tissue. We carried out qPCR analysis in 20 µl reactions with 11 replicates of each concentration, which ranged from 1.6 ng to 1.6 fg as quantified using a Qubit Fluorometer (Invitrogen v. 2.0). We estimated the 95% limit of detection (LOD) of the assay by fitting a 3-parameter log-logistic dose–response curve to the resulting concentration and detection data following Klymus et al. (2020).

Results

Our *in silico* specificity test evaluated sequences of 10 relatives co-occurring in the US northeast and showed evidence that there are sufficient polymorphisms in the primer and probe regions such that they would not cross-amplify with our designed assay (Appendix 1: Fig. A1). For our *in vitro* specificity test, we ran extracted DNA from seven species (*Agrilus arcuatus*, *Agrilus bilineatus*, *Phaenops fulvoguttata*, *Spectralia gracilipes*, *Dicerca divaricata*, *Dicerca lurida* and *Actenodes acornis*) through our EAB COI qPCR assay and all samples returned negative amplification results. All species tested are order Coleoptera and family Buprestidae. Of all the species tested for assay specificity, the only one which is known to co-occur on ash (*Fraxinus* spp.) is *Agrilus subcinctus*.

The modelled 95% LOD of our EAB qPCR assay (Klymus et al. 2020) was 16 fg per reaction (95% CI = [6.25, 130]) assuming three technical replicates were performed. The lowest concentration of EAB genomic DNA detected from our serial dilution was 1.6 fg.

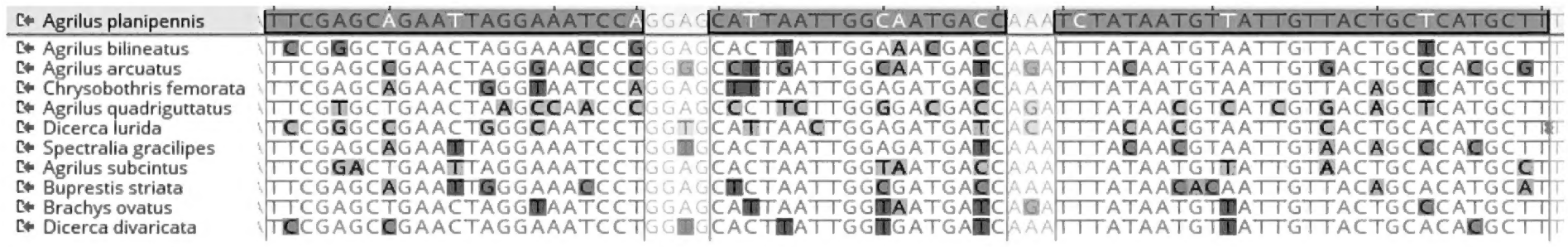

Figure A1. Based on published genetic data in conjunction with species sequences we generated, we created a *Geneious Prime* sequence alignment to analyse how many base pair polymorphisms existed between each of 10 co-occurring Buprestid species in the primer and probe regions we designed for our EAB COI qPCR assay (highlighted in grey). The sequences listed are consensus sequences based on a combination of those we generated ourselves as well as those accessed on GenBank.

We break down below the number and source of sequences used to generate these consensuses as follows:

Agrilus bilineatus: 1 sequence we generated + HQ582712, HQ582713, MF286166, MF805329, MH159018, MH159107

Agrilus arcuatus: MF286192, MF286193, MF805139, MF805156, MF805159, MF805179, MF805196, MF805245, MF805295
Chrysobothris femorata: 1 sequence we generated + JF888345, KR126263, KR481996
Agrilus quadriguttatus: 1 sequence we generated
Dicerca lurida: 2 sequences we generated + MG057907
Spectralia gracilipes: 1 sequence we generated + KM364375, KM847081
Agrilus subcinctus: 1 sequence we generated
Buprestis striata: KR482483
Brachys ovatus: HQ582477
Dicerca divaricata: AY165645, MG054990

Appendix 2

Accounting for potential contamination

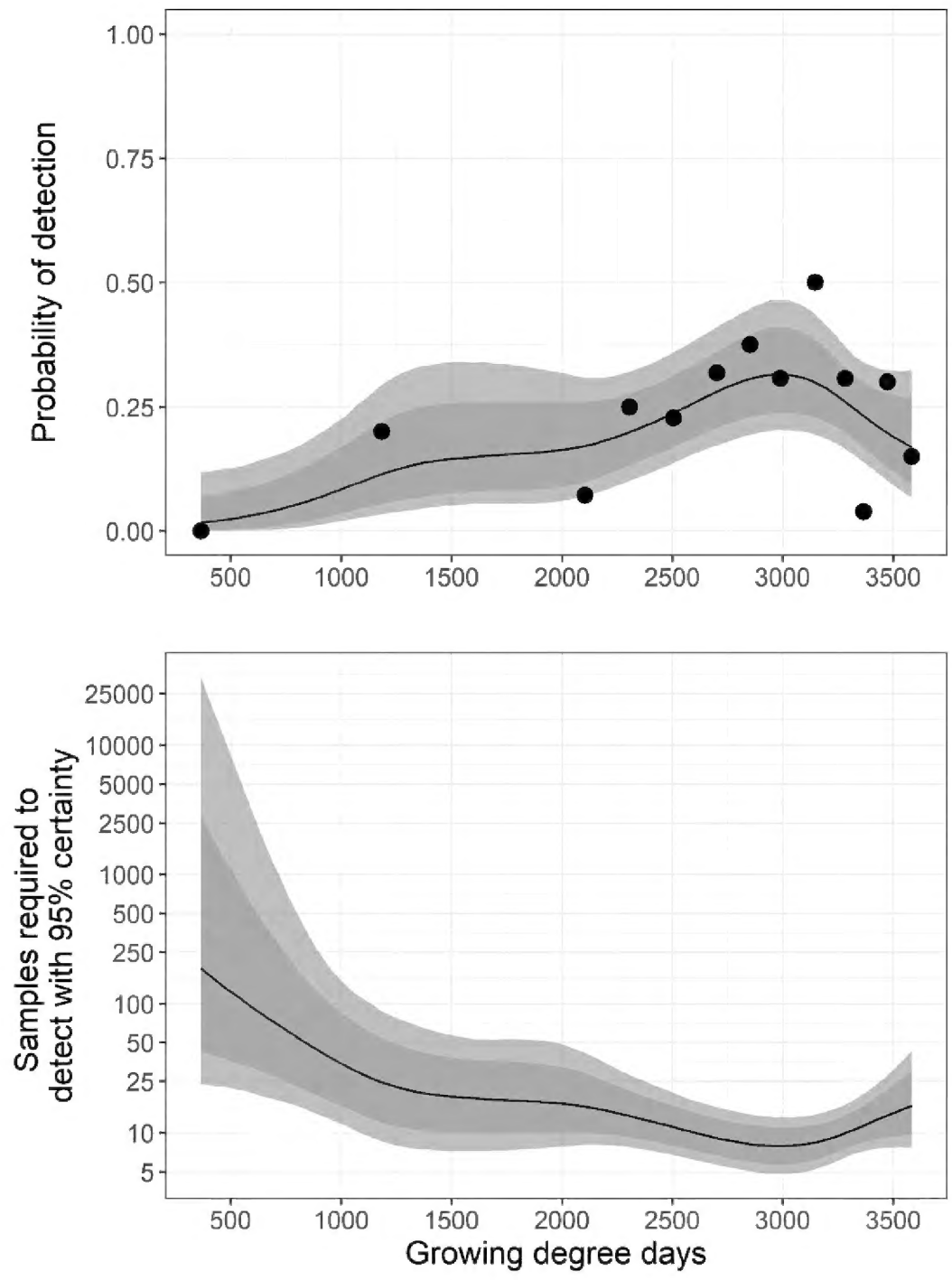

Figure A2. This plot shows the same model output as in Fig. 2, but with detections defined more conservatively, as samples with at least two technical replicates successfully amplifying. Top: probability of detecting eDNA from EAB using the tree core sampling method at sites in New Jersey, USA over time. Bottom: the number of ash tree core samples needed per tree to ensure 95% confidence in detection of EAB larval presence within a tree. Time is represented as growing degree day accumulation, base 50 °F (10 °C). Black lines represent the posterior medians from a Bayesian general additive model; grey shading indicates 80% and 95% credible intervals. Black circles show the proportion of samples with detections on each sampling date calculated from the raw data. The GAM model was fitted and used default vague priors in accordance with Bürkner (2017).